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(54) Title: VACCINES BASED ON HEPATITIS B SURFACE ANTIGEN			
(57) Abstract Novel antigen are presented which are useful in vaccine formulations for the prophylactic treatment of a range of infectious diseases. The antigens comprise a hybrid polypeptide, one part being the S antigen of the hepatitis B virus, the other being a heterologous antigen, such as gD from HSV. The two antigens are linked by chemical spacers through a native sulphhydryl group present on the surface of the S antigen.			

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Vaccines based on Hepatitis B surface antigen

The present invention relates to improved immunogens which comprise an antigenic polypeptide chemically conjugated to 5 hepatitis B surface antigen (HBsAg). The invention further relates to novel vaccines and their use.

Proteins or synthetic peptides comprising epitopes of different viruses represent potential immunogens for use in 10 vaccines against the infectious diseases caused by the respective viruses. However, such polypeptides frequently require a combination of carriers and adjuvants to become sufficiently immunogenic for consideration as vaccines.

15 It would appear that correct antigen presentation is the key requirement for an effective subunit vaccine and Valenzuala et al. (Biotechnology, 1985, 3, 323-326) have concluded that a good immunogen should have the maximum number of its epitopes properly exposed. This requirement was stated by 20 these authors to be difficult to achieve by random chemical coupling of antigens to a carrier molecule. Accordingly a new approach was tried in which HBsAg was used as a carrier and first antigen molecule and the gene encoding a second antigen was recombined with the gene for HBsAg so that the 25 second antigen was assembled into and presented on the surface of the HBsAg particle. Using an N-terminal fusion with a truncated form of the HBsAg middle protein Valenzuala et al. (loc.cit.) observed particle assembly of a Herpes Simplex Glycoprotein D - HBsAg hybrid polypeptide. The 30 glycoprotein D (gD) epitopes were found to be presented in a repetitive fashion at the at the surface of the particle, thereby greatly enhancing the immunogenicity of the gD component.

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More recently it has been shown that the major repetitive epitope of P. falciparum circumsporozoite (CS) protein could be fused to HBsAg. Immunogenicity of the hybrid particles was found to be superior to that of an equivalent monomeric 5 CS antigen (Rutgers *et al.*, Biotechnology, 1988, 6, 1065).

Vaccines prepared from hybrid immunogenic particles comprising HBsAg protein are also described in European Patent Application Publication No. 0 278 940. In all cases the hybrid particles were obtained by gene fusion rather 10 than chemical coupling techniques.

Despite the emphasis on gene fusion as a means to designing polyvalent vaccines it has now been found, surprisingly, that effective immunogenic molecules can be prepared by 15 chemical cross-linking of HBsAg protein (or a suitable fragment thereof) having at least one free sulphhydryl group on its surface with another antigen.

Accordingly the present invention provides an immunogenic 20 hybrid polypeptide comprising a first polypeptide component which is HBsAg or fragment thereof displaying the antigenicity of HBV surface antigen, covalently linked via a native sulphur atom in the first polypeptide component to a second polypeptide component.

25

An advantage of the invention is that an antigen may be coupled to the HBsAg particle with cross-linking agents without impairing the immunogenicity of the HBsAg or fragment thereof as defined hereinabove. Furthermore it is 30 possible by the present invention to conjugate the second polypeptide antigen with a vector (the HBsAg particle) which is able to direct its processing via a non-endosomal route. In this way the second antigen can become associated with MHC I antigens and be recognised as such by cytotoxic 35 lymphocytes. Finally chemical coupling allows a higher degree of freedom with regard to antigen (epitope) density

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on the HBsAg particle and also the possibility of using non-immunogenic spacers whereby the distance from the attachment point to the particle can be varied at will.

5 In one aspect, the hybrid polypeptide of the invention may be represented by formula (I):



10 in which the group P^1-S- is HBsAg or fragment thereof displaying the antigenicity of HBV surface antigen bonded via a native sulphur atom;

15 P^2 is a second antigenic polypeptide; and

X is either:

a) a group $-A-NH-$ wherein A is a spacer group and $NH-$ is the residue of an amino group present in the side chain of 20 an amino acid in P^2 ;

or optionally if P^2 comprises a cysteine residue and a hydrophobic anchor group:

25 b) the sulphur atom of the said cysteine residue present in P^2 .

Preferably P^2 is selected from gD₂t from HSV or is a peptide capable of binding to HIV neutralising antibodies and 30 corresponds to the neutralising domain of the V₃ loop from gp120; and

The V₃ loop peptides utilised in the present invention are preferably between 10 to 21 amino acids in length and 35 comprises a β turn flanked by at least one and preferably at least two amino acid on both sides of the β turn sequences.

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Preferably the sequence corresponds to the sequence 310 to 328 of the gp160 protein.

The following peptide is preferred.

5

TYR THR ARG LYS SER ILE ARG ILE GLN ARG GLY PRO GLY ARG
ALA PHE VAL THR ILE GLY

The C-terminal tyrosine is optional, as its primary function 10 is to allow labelling with radioactive Iodine.

An important advantage of the hybrid particles according to the present invention is their ability to induce cellular immunity mediated by T lymphocytes and in particular the 15 ability to induce cytotoxic T lymphocytes (CTL) responses.

It will be appreciated that formula (I) is a simplified representation since the second antigen polypeptide P^2 will not normally be bonded to P^1 solely through one sulphur 20 atom. Furthermore, P^1 is preferably in particulate form as described below.

It is essential to the invention that the HBsAg or fragment thereof used as the first polypeptide component has at least 25 one free thiol on its surface (e.g. may be represented as P^1-SH) and to this end it is important that the HBsAg or fragment thereof is obtained in the correct manner.

Commercially available vaccines against HBV comprise 30 Hepatitis B virus surface antigen (HBsAg) either in native or recombinant form. The authentic Hepatitis B virus surface antigen can be recovered from plasma of infected individuals as a particle of about 22nm comprised of two

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proteins known as P24 and its glycosylated derivative GP28, both of which are encoded by the 226 amino acid coding sequence on the HBV genome known as the S-protein coding sequence or HBV S-gene; see Tiollais *et al.*, Nature, 317

5 (1985), page 489 and references therein. The complete amino acid sequence of, and nucleotide sequence encoding, HBsAg is given in Valenzuela *et al.*, Nature, 280 (1979), page 815. The numbering system used by Tiollais *et al.* (loc cit.) to define nucleotide and amino acid positions is used herein.

10

Insertion of HBV S-gene coding sequences under the control of yeast promoters on expression vectors to enable expression of HBsAg in S. cerevisiae for vaccine production has been described by, for example, Harford *et al* in 15 Develop. Biol. Standard. 54: page 125 (1983), Valenzuela *et al.*, Nature 298, page 347 (1982) and Bitter *et al.*, J. Med. Virol. 25, page 123 (1988). Expression in Pichia pastoris has also been described by Gregg *et al.*, Biotechnology, 5 (1987), page 479 (see also European Patent Application 20 Publication No. 0 226 846) as has expression in Hansenula polymorpha (see EP-A- 0 299 108).

Not all the above methods give HBsAg suitable for use in the present invention since recombinant HBsAg produced in 25 mammalian cells or yeast by the method of Valenzuela and others does not have available free SH groups; it is believed that the cysteine residues of HBsAg are all involved in the formation of disulphide bonds (Wampler *et al.* (Proc.Natl. Acad. Sci. U.S.A. 1985, 82, 6830-6834 and 30 references therein)).

EP-A-0 135435 (assigned to Merck and Co.) describes a method for efficiently converting the non-disulphide bonded HBsAg antigen into a fully intermolecular disulphide bonded 35 particle, alleged to be ten times more immunogenic than the

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antigen which has not been so treated.

However, recombinant HBsAg as produced in S. cerevisiae by SmithKline Beecham Biologicals for the preparation of the 5 vaccine Engerix-B* (Harford *et al.* loc.cit.) does have an average of four free cysteines per S monomer and does form particles and this has been found to be at least as immunogenic as the fully intermolecular disulphide bonded particle. It will be apparent that when HBsAg is in this 10 form then free cysteines provide one or more native sulphur atoms which can be utilised for coupling the second polypeptide. It will also be appreciated that preferably the HBsAg forms a particle, typically a lipoprotein particle.

15

It is to be understood that the first polypeptide component in the hybrid according to the present invention may comprise all or part or parts of the HBsAg precursor protein encoded by the coding sequence which immediately precedes 20 the HBV-S gene on the HBV genome referred to herein as the Pre-S coding sequence.

The pre-S coding sequence normally codes for 163 amino acids (in the case the ay HBV sub type) and comprises a pre-S1 25 coding sequence and a Pre-S2 coding sequence. The latter codes for 55 amino acids and immediately precedes the S-protein coding sequence (see EP-A-0 278 940 for further details).

30 In one preferred aspect the first polypeptide component P^1 is the HBsAg S-protein having one or more, preferably up to 4, sulfhydryl groups on its surface.

* Engerix-B is a Trade Mark

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From the foregoing it will be appreciated that the first polypeptide component is preferably prepared by recombinant DNA techniques, for example by expression in S. cerevisiae as described by Harford *et al* (loc.cit.), i.e. corresponds to or comprises the HBsAg antigen present in the commercial vaccine Engerix-B*.

In another aspect the first polypeptide component may comprise a fragment or truncate of the HBsAg S-protein 10 provided the said fragment has at least one free sulphhydryl group on its surface available for coupling to the group X, and provided that particle assembly is not adversely affected.

15 In yet another aspect the first polypeptide component may be part of a composite particle comprising at least two polypeptides corresponding to part or all of a protein having the biological activity of one of the hepatitis B surface antigens wherein the particle presents at least two 20 antigenic determinants provided by the S-protein, pre-S2-protein or pre-S1-protein, said particle optionally containing host specific lipids, as described in copending European Patent Application No. 0414 374.

25 An example of such a composite particle may be represented by (L,S) where L is the large protein of HBsAg (including the pre-S1, pre-S2 and S coding sequences as hereinabove defined) and S is the HBsAg S-protein.

30 In yet a further aspect the first polypeptide component may be a modified L protein of HBsAg as described in copending European Patent Application No. 0414 374 wherein the modified L protein comprises a modified hepatitis B virus large surface protein comprising an amino acid sequence

35

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encoding the L protein characterized by a modification in at least one of the following sequences: a sequence sensitive to protease digestion, a sequence necessary for myristylation, a sequence necessary for N-linked 5 glycosylation, a sequence necessary for O-linked glycosylation, and a sequence necessary for binding of human serum albumin.

In a preferred aspect the modified L protein may be 10 represented by L* wherein L* has an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L protein.

In another preferred aspect the first polypeptide in the 15 compound of the present invention may be part of a composite particle (L*,S) wherein L* and S are as hereinabove defined. Such composite particles may be prepared as described in copending European Patent Application No. 0414 374.

20 The second polypeptide P² is an antigen useful in the preparation of a polyvalent vaccine and may be of any suitable structure.

Specific antigens for P² include the recombinant DNA 25 envelope protein gD of Herpes Simplex Virus (HSV), particularly the truncated form of gD known as gD_{2t} from HSV2. Other antigens which may be mentioned are malaria antigens, particularly those derived from the circumsporozoite protein, or antigens derived from HIV 30 envelope protein.

The human immunodeficiency virus (HIV) has been identified as the causative pathogen of acquired immunodeficiency syndrome (AIDS). Like other members of the retroviral family, the 35 genes encoding the major structural proteins of the virus

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are defined within the genome by env (viral envelope glycoprotein) and gag (core proteins) genes.

The envelope glycoprotein is known as gp120. After 5 infection with the AIDS virus, human beings develop antibodies against this glycoprotein. In many patients neutralising antibodies are produced although, it is also known that different HIV isolates exhibit a diverse array of sequence variation, particularly in the envelope gene.

10

Peptides from the major neutralising domain located in the envelope protein gp120 in a region known as the V₃ loop are known to bind to neutralising antibodies generated in vivo. Nonetheless in order for those peptides to generate 15 neutralising antibodies, correct presentation of the peptide is required. The present invention achieves this, by presenting such peptides on the surface of HBsAg in the manner described herein.

20 In certain circumstances, the second polypeptide may comprise a hydrophobic anchor group (a hydrophobic 'foot') which may be naturally or synthetically attached to its amino terminal.

25 Suitable hydrophobic anchor groups include fatty acid residues such as myristoyl, palmitoyl and lauryl.

The advantage of having such a hydrophobic anchor group in the second polypeptide component is that it can, via 30 hydrophobic interaction, become embedded in the lipidic membrane associated with the first polypeptide component.

If the second polypeptide component also comprises an accessible cysteine residue the thiol group of the said 35 cysteine can then, by spontaneous oxidation, form an intermolecular disulphide bond with a native thiol group in the first polypeptide component, thereby contributing to the

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stability of the formed complex. In such a case it is unnecessary to use a chemical cross linking agent to form the hybrid according to the invention (i.e. the group X as hereinabove defined may represent a sulphur atom which is native to P^2 as hereinabove defined).

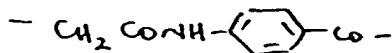
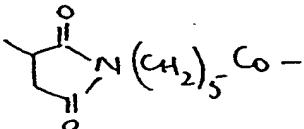
The linker group A as hereinabove defined represents a linear spacer group bonded at one end to a native sulphur atom of the first polypeptide and at the other end to the second polypeptide P^2 via an amino acid containing side chain in P^2 .

Examples of the group A are substituted C_2-C_{10} alkanes or linear polymers such as polyethylene glycol.

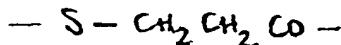
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Particular groups A include:

20



and



25

The present invention further provides a process for the preparation of a hybrid polypeptide of formula (I):



30

wherein P^1 , P^2 and X are as hereinabove defined which process comprises the steps of

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a) when X is an a group -ANH-:

i) reacting the second polypeptide P^2 with a compound of formula (II):

5



wherein Y is a group capable of reacting with a native thiol group in the first polypeptide; B is a group which is 10 specific for one or more amino acid side chains on the second polypeptide P^2 and A is as hereinabove defined; and thereafter

ii) reacting the product with the first polypeptide 15 P^1 -SH; or

b) when X is S and F^2 comprises a hydrophobic anchor group and a cysteine residue:

20 i) mixing the first polypeptide and second polypeptide in aqueous solution; and

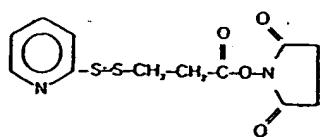
ii) allowing an intermolecular disulphide bond between P^1 and P^2 to form by spontaneous oxidation.

25

Heterobifunctional reagents of formula (II) are known in the art and include N-succinimidyl 3-(2-pyridylthio)propionate [SPDP; (III)], succinimidyl 6-maleimidyl hexanoate [EMCS (IV)], and N-succinimidyl 4-(iodoacetyl)amino benzoate [SIAB 30 (V)].

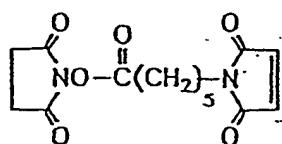
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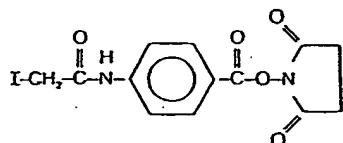
(III)

10



(IV)

15



(V)

In method a) according to the above process steps i) and ii) may be carried out standard conditions as known in the art for cross-linking proteins, and the final product may be 20 purified by, for example, preparative high pressure liquid chromatography.

Particular hybrids which are the subject of the present invention include:

25

HBsAg S protein crosslinked to gD₂t of herpes simplex virus 2 using reagent (V);

HBsAg S protein linked to LCF6 as hereinbelow defined via an 30 intermolecular disulphide bond, and HBsAg S-protein linked to V₃ peptides from HIV gp120;

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Typically the ratio of the second polypeptide to the first polypeptide will be in the range 0.1 to 1.0 molecules per monomer. Although in the case of V₃ peptides this may be in the range of 1.0 to 4 molecules per monomer.

5

In a further embodiment of the present invention, the polypeptide P¹ may be mixed with a mixture of V₃ peptides. The resulting fusion will then have different V₃ peptide attached to the surface of P¹.

10

In the present case the efficiency of the reaction is such that approximately forty peptides are linked to each particle it being understood that the hybrids according to the invention are preferably in particulate form.

15

In a further aspect the invention provides a vaccine composition comprising an immunoprotective amount of a hybrid polypeptide according to the invention together with a conventional carrier or adjuvant.

20

A preferred adjuvant, according to the present invention is de - 3 - 0 - acylated monophosphorylated lipid A (3D-MPL) in a suitable carrier. This adjuvant system provides high neutralising antibody titres.

25

3D-MPL may be obtained by the methods described in U.K. patent No. 2,211502 (RIBI).

In the case of utilising HIV peptides the present inventors 30 have found that exceptional results may be achieved by first adsorbing the HBsAg-V₃ peptide conjugate of the present invention on to alum and then admixing with 3D-MPL.

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Additionally, oil in water emulsions containing 3D-MPL provide excellent results. The oil in water emulsion formulation provided by the present invention most 5 preferably comprises, 3D-MPL, squalane, pluronic L -121 and phosphate buffered saline.

The emulsion is preferably passed through a microfluidizer to provide submicron particles in the emulsion. This 10 enhances the activity of the formulation.

Alternatively vaccines containing the hybrid polypeptide according to the invention are prepared by conventional techniques and will contain an immunoprotective amount of 15 the hybrid preferably in buffered physiological saline and admixed or adsorbed with any of the various known adjuvants including aluminium hydroxide and aluminium phosphate. By "immunoprotective" is meant that enough of the hybrid is administered to elicit a sufficient protective antibody or 20 cell mediated immune response to confer protection against an infectious agent without serious side effects. The amount of hybrid to be administered will depend on whether the vaccine is adjuvanted and will generally comprise between 1 to 1000 mcg of total protein, for example 1 to 200 25 mcg total protein, more preferably 5 to 40 mcg total protein. The amount and number of doses to be administered can be determined in standard dose range studies involving observation of antibody titres and other responses in subjects.

30

The hybrid polypeptide according to the invention may also be mixed with other antigens such as composite HBsAg particles containing all or part or parts of the PreS1 or PreS2 polypeptides for vaccine formulation. It may also be 35 mixed with fused or other chemically synthesised hybrid

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HBsAg particles carrying epitopes from proteins from other organisms and with other immunogens to form multivalent vaccines. Vaccine preparation is generally described in "Vaccines", edited by Voller et al, University Park Press, 5 Baltimore, MD, U.S.A., 1978.

Accordingly in a further aspect, the invention provides a method of immunising a subject against viral infection which comprises administering to a subject in need of such 10 immunisation an effective amount of a vaccine composition according to the invention.

The following examples illustrate the invention.

15 Examples

A. Determination of free sulphydryls on the recombinant HBsAg particle

20 A.1. Material and Methods

a) BNP Method

A method for identifying cysteine-containing peptides in 25 proteins was applied using 2-bromoacetamido-4-nitrophenol (BNP) to introduce an easily detectable probe. The formation of a covalent bond between the protein sulphydryl group and the acetamido moiety of BNP introduces a chromophore with an absorbance maximum at 410 nm. The 30 modified protein can then be cleaved with appropriate proteases and the resulting peptides separated by chromatographic methods. Monitoring the effluent at a single wavelength (405 nm) provides a rapid and simple method for detecting and isolating only those peptides which 35 contain cysteine residue(s).

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Materials

HBsAg was from SmithKline Biologicals. NH_4HCO_3 , 2-bromoacetamido-4-nitrophenol (BNP) and chymotrypsin were 5 from Sigma. Trifluoroacetic acid (TFA) and acetonitrile were from Baker (HPLC grade). N,N-dimethylformamide (DMF) was from Janssen Pharmaceutica.

Protein labelling

10

Protein (\pm 1 mg) was dialyzed against labelling buffer (0.1 M Tris-HCl, EDTA 2 mM, Urea 8M, pH 8.6). 2.5 mg of BNP (dissolved in DMF) per mg of protein was added to the denatured protein. After 90 minutes of incubation at room 15 temperature, unreacted BNP was removed by extensive dialysis against NH_4HCO_3 100mM, pH 8.5.

Digestion of modified protein

20 Duplicate addition of chymotrypsin (t_0 and $t_0 + 4$ hours) (2% W/W) was made. Incubation was then carried out at 37°C overnight. The digested protein was stored at 4°C.

HPLC separation of the peptides was accomplished on a Waters 25 600 HPLC system fitted with a 250 mm x 4.6 mm Vydac C₄ reverse-phase column. The column was equilibrated with HPLC buffer (NH_4HCO_3 100 mM, pH 8.5) prior to injection of the sample (\pm 1 mg). Peptides were eluted with a linear acetonitrile gradient from 0 to 50% in 50 minutes. The 30 elution was monitored at 405 nm and at 224 nm on a Waters 490 Multiwave Detector.

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Sequence analysis of the BNP peptides

Dried sample was redissolved in 60 μ l TFA 6%, and applied on the glass fiber membrane of the sequenator. The membrane 5 was preferably treated with Biobrene. Edman automatic sequential degradation was performed with a liquid phase sequenator (Applied Biosystem 477A) coupled with an amino acid analyser (Applied Biosystem 120A).

10 The program used is analogous to that of Hewick et al. J. Biol. Chem., 1981, 256, 7990-7997. Phenylthiohyantoin derivatives of cleaved amino acids were identified by RPLC, following the gradient system described by Hunkapiller and Hood, (Methods Enzymol. 1983, 91, 486-493).

15

b) PDS Method

20 The method of Grasetti et al (Arch. Biochem. Biophys., 1967, 119, 44-49) was followed. Upon reaction of protein thiols with 2,2'-dithiodipyridine (thiol disulfide exchange reaction) 2-thiopyridine is liberated and measured at 343 nm ($\epsilon_{343 \text{ nm}} = 8.08 \times 10^3 \text{ M}^{-1}$).

Materials

25

HBsAg was from SmithKline Biologicals. Dithiothreitol (DTT) and 2,2'-dithiodipyridine (PDS) were from Serva. PD 10 gel filtration columns were from Pharmacia.

30 Assay procedure

Protein (\pm 750 μ g/ml) was dialyzed against buffer (Urea 8M, EDTA 2 mM, 0.1 M Tris-HCl, pH 8.5). PDS (25 M in excess, dissolved in ethanol) was added to the denatured protein.

35 After one hour of incubation at room temperature, excess of

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PDS was removed by gel filtration (PD 10 column). Absorbance of the modified protein was measured at 280 nm. Addition of DTT (10 mM final concentration) was made to measure the absorbance of the free thiones at 343 nm.

5

c) Carboxymethyl-cysteine determination

Materials

10 Iodoacetamide was from Merck. HBsAg was used from SmithKline Biologicals.

Carboxymethylation

15 Protein (1 mg/ml) was dialyzed against buffer (Tris 100 mM, EDTA 2mM, pH 8.0) with or without Urea 8M. The S-carboxymethylation of the thiol groups was performed by addition of iodoacetamide (100 moles per mole of sulphhydryl groups) for 20 minutes, in the dark at room temperature.

20

Amino acid analysis

Hydrolysis: Aliquots of protein (\pm 100 μ g) were dried in conic hydrolysis tubes, in a Speed Vac Concentrator.

25 Hydrolysis was performed by addition of HCl 6N (500 μ l) containing 0.5% phenol, at 110°C during 24 hours. Samples were done in triplicate.

After cooling, hydrolysate was evaporated to dryness, washed 30 in 500 μ l water, and dried again. Having been dissolved in 200 μ l of 0.2 N pH 2.2 citrate buffer and filtered on a 45 μ m membrane, 50 μ l of each sample is injected on the analyser column.

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Amino acid analysis: was performed with an Automatic Analyser (Alpha Plus-LKB 4151) on a polystyrene sulfonated column. A three buffer elution system was used to separate the different amino acids:

5

1. citrate buffer 0.2 N pH 3.2 at 54°C
2. citrate buffer 0.2 N pH 4.25 at 56°C
3. borate buffer 0.2 N pH 10.0 at 90°C

10 Amino acid detection was made by post-column reaction with ninhydrin and by colorimetric measurement at 440 and 570 nm. For quantification, optical densities at 440 and 570 nm are added together and chromatographic data integration were performed on a Shimadzu CR-A3 integrator.

15

A.2. Results

a) BNP Method

20 After modification of the free thiols by BNP and chymotryptic digestion, peptides containing the BNP label were separated by RPLC and identified by sequencing, following the procedure of Gardner *et al.* Anal. Biochem., 1987, 677, 140-144.

25

HPLC analysis of 5 different HBsAg chymotryptic digestions yielded 5 well separated major peaks indicating a reproducible oxidation state for all batches. The sequences of the BNP labelled peptides allow the localization of 4 thiol groups (cys 48, 65, 121 and 124).

30 The identical analysis was performed on HBsAg produced in mammalian cells. No BNP labelled peak could be detected indicating that no free cysteines were available.

35

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b) PDS Method

Four thiol groups per monomer of HBsAg were detected after application of the PDS method on 3 different batches of 5 HBsAg particles from SmithKline Biologicals (Batches 1 to 3 in Table 1 below) No SH free per monomer could be detected in a batch of HBsAg expressed in Chinese hamster ovary (CHO) cells (Batch 4).

10 Table 1:

PDS Method: FREE THIOL GROUPS DETERMINATION

	BATCH NUMBER	SH FREE PER MONOMER
15	1 (yeast)	4.38
	2 (yeast)	3.18
	3 (yeast)	4.2
	4 (CHO cells)	0

25 c) Carboxymethyl-cysteine determination

Cysteines in HBsAg particles were carboxymethylated with or without denaturing agent (Urea 8M).

30 Subsequent amino acid analysis showed the presence of 3 carboxymethylcysteine per monomer of HBsAg in both cases.

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B. Examples of coupling antigens to the HBsAg particle

B.1. EXAMPLE 1: Covalent coupling of the glycoprotein D of Herpes Simplex 2 Virus to a particulate carrier

5

B.1.1. Introduction

The glycoprotein D of HSV 2 (gD₂t) expressed in CHO cells (Lasky and Dowbenko DNA, 1984, 3(1), 23-29) is covalently 10 coupled to a recombinant HBsAg particle containing free SH groups.

B.1.2. Materials and Methods

15 a. Agents

• 5,5'Dithiobis 2-nitrobenzoic acid (Ellman's reagent or DTNB) and N-succinimidyl(4-iodoacetyl)- aminobenzoate (SIAB) were purchased from PIERCE.

20

• 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was obtained from SERVA.

• Recombinant gD₂t was expressed in CHO cells and 25 purified by SmithKline Biologicals.

• gD₂t was iodinated by the enzymobeads method of PIERCE.

30 . HBsAg particles were produced by SmithKline Biologicals.

b. Methods

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b.1. Characterization of gD₂t

b.1.1 Quantitative determination of sulphhydryl groups:

5 To 150 μ l of gD₂t (23 μ M in Na₂HPO₄ 0.02 M pH 7) 1 ml of DTNB (3.28 mM in Na₂HPO₄ 0.04 M pH 8) is added.

After 5 min, the optical density at 412 nm is determined against a blank lacking protein. An extinction coefficient 10 of $1.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ is used to calculate the concentration of sulphhydryl groups reacting (Ellman, Arch. Biochem. Biophys., 1959, 82, 70).

b.1.2 Quantitative determination of lysine residues:

15

50 μ l of TNBS/H₂O 24.5 mM are added to 50 μ l of gD₂t (58 μ M in Na₂HPO₄ 0.02 M pH 7) diluted in 200 μ l of borate buffer (0.05 M Na₂B₄O₇ adjusted to pH 9.5 with 0.05 M NaOH).

20 After 3 hours in the dark at room temperature the change in absorbance at 367 nm is followed against a blank without protein.

The extent of trinitrophenylation is calculated on the basis 25 of an $\epsilon_{367 \text{ nm}} = 1.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Plapp et al., J. Biol. Chem. 1971, 246 (4), 939-945).

b.2. Activation of gD₂t with SIAB

30 100 μ l of gD₂t (23 μ M in Na₂HPO₄ 0.02 M pH 7) + 100 μ l gD₂t I¹²⁵ are incubated for 30 min at 37°C with 2 μ l SIAB (25 mM in DMSO) which corresponds to a molar ratio succinimide/lysine of 2. The excess of cross-linker is eliminated by dialysis (2 hours against Na₂HPO₄ 0.02 M pH 8) 35 and the reaction mixture is concentrated to 100 μ l by

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ultrafiltration on an YM 10 centricon.

b.3. Coupling to HBsAg particle

5 gD₂t (100 μ l), concentrated (1 mg/ml) and SIAB-activated, is incubated with 53 μ l of HBsAg particles (1 mg/ml in Na₂HPO₄ 10 mM pH 7.2, NaCl 150 mM) for various times at 37°C.

The initial molar ratio gD₂t/S monomer is 1/1.

10 The particulate gD₂t is purified by a 1.5 M CsCl gradient (45 hours, 65000 rpm in a 70.1 Ti rotor).

b.4. Quantification of gD₂t coupled per particle

15 50 μ l of water are added to a vial of enzymobeads. After one hour, 50 μ l of Na₂HPO₄ 0.2 M pH 7.2, 25 μ l gD₂t (1 mg/ml), 0.5 mCi NaI¹²⁵ (Amersham) and 25 μ l 1% β -D-Glucose are added.

20 After 20 min at room temperature, the reaction is completed and the iodinated protein is separated from free iodine by chromatography on DOWEX Ag 1 x 8 resin saturated by BSA 1%.

The specific activity of the gD₂t involved in the coupling 25 may be determined by the radioactivity detected in the mixture of labelled and non-labelled gD₂t. The amount of gD₂t coupled to particles may be determined by this specific activity.

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B.1.3. Results

a. Characterization of gD₂t

5 a.1. Quantitative determination of sulphydryl groups No free thiol is detected on the gD₂t by DTNB. This result fits the aminoacid sequence of the protein. The truncated gD₂t molecule used (283 aa) contains 6 cysteine residues, each involved in disulfide bridges that constitute 10 discontinuous epitopes. Therefore, gD₂t is an ideal molecule for the activation step with a heterobifunctional cross-linker without risk of homopolymerisation.

a.2. Quantitative determination of lysine residues

15

The number of free amino groups are detected by TNBS either on the native or on the SIAB activated gD₂t. The number of free lysines decreases as a function of the excess of SIAB.

20 With a molar ratio SIAB/lysine of 2, four residues are activated.

The number of detected lysines on the native protein (10) is close to the number determined in the amino acid sequence 25 (11).

b. Activation of gD₂t by SIAB

After an activation of 30 min at 37°C with a molar ratio of 30 SIAB/lysine of 2, the absence of homopolymers of gD₂t is checked by gel filtration.

On a TSK 3000 column, the homopolymers elute in the void volume (8 min) and the monomeric gD₂t has a retention time 35 of 15 min.

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Despite the absence of cysteine residues in the protein, formation of homopolymers is observed when gD_2t is activated at a concentration of 2.5 mg/ml. An aspecific reactivity of the halogen in SIAB for lysine, methionine or histidine residues may explain this phenomenon (see Means and Feeney, Chemical Modifications of Proteins; Holden Day publ., 1971, page 107).

Initial protein concentration is a decisive factor in homopolymerisation events. If gD_2t is activated at a concentration of 0.5 mg/ml, the formation of homopolymers decreases from 50 to 10%.

c. Coupling to HBsAg particle

15

The HBsAg- gD_2t conjugate obtained after a 30 min, 2 hrs or over night incubation at 37°C is purified by CsCl gradient. The gD_2t homopolymers have a different density to the carrier and do not contaminate the conjugate. The yield of coupling increases with time as shown in Table 2. 0.2 gD_2t molecules are coupled per S monomer (twenty per particle) as calculated by radioactivity detected in the particle's density area.

25 Table 2: Influence of incubation on the yield of coupling

	Time	gD_2t/S monomer
30	30 min	0.08
	2 hrs	0.14
	15 hrs	0.20

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B.2. Example 2

B.2.1. Incorporation of LCF6 [Lauroyl-Cys-Tyr-Gly-Gly-(NPNA)6] into HBsAg particles

5

10 μ l of LCF6 solution (1 mg/ml in phosphate 10 mM pH 7, NaCl 150 mM) are incubated with 10 μ l of a solution of yeast expressed HBsAg particle (ex SmithKline Biologicals) at 1 mg/ml in phosphate 10 mM pH 7, NaCl 150 mM overnight at 10 37°C.

B.2.2. Assay

Quantitation of the HBsAg-LCF6 hybrid is performed by a 15 sandwich ELISA test using as coating antibody a polyclonal IgG against HBsAg at a concentration of 5 μ l/ml and as detecting antibody a biotinylated monoclonal IgM directed against the repeat sequence of LCF6.

20 An antigen R16 HBsAg solution (a recombinant repeat Malaria-HBsAg particle) of known concentration is used as standard. This ELISA presents the advantage of monitoring the hybrid without prior separation of the free peptides. The results are expressed in μ g/ml equivalent R16 HBsAg.

25

B.2.3. Implication of a covalent bond in the LCF6 incorporation into HBsAg particle

To evaluate the relative important of the disulfide bridge 30 and of the hydrophobic foot, the incorporation of the peptide without cysteine and of the peptide without the lauroyl group is compared. The ELISA shows that the lipopeptide lacking cysteine (LF6) fails to incorporate into the HBsAg particle. The peptide without the lauroyl group 35 (CF6) is nearly ineffective. Therefore the synergy between

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hydrophobic and covalent interactions is critical for the peptide's incorporation into HBsAg particle.

The results from LCF6 incorporation into different HBsAg particles with variable oxidation state were compared.

The lipopeptide is incubated with classical HBsAg particle (\pm 4 free SH groups/S monomer), with more oxidized particles (1 free SH group/S monomer), or with totally oxidized particles (no free SH group; particles synthesized in CHO cells).

These results indicated that a disulfide bridge between LCF6 and S monomer stabilizes the peptide incorporation. The ELISA for anti-HBsAg-LCF6 show decreasing coupling as a function of an increasing oxidation state of HBsAg particles.

The existence of covalent linkage between LCF6 and HBsAg particle is also demonstrated by analysis on SDS-PAGE.

Bands at 26 and 50 Kd appear in non-reducing conditions.

These bands correspond to the LCF6 peptide coupled to the S monomer and dimer.

In contrast, in reducing conditions, these bands disappear.

B3. Example 3 : Incorporation of V₃ peptides into HBsAg particles

3.1. V₃ loop structure

The peptide used represents the sequence from amino acid 310 to 328 (G. Larosa, Science 1990, 249-932) comprised in a disulfide bridged loop in the third variable region of the external protein gp120. Its conservation is over 80% in 9

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out of 14 positions in the central portion and its predicted structural motif is of the type β strand-type II β turn- β strand- α helix.

5 The primary structure of the peptide can be represented by the following

TYR THR ARG LYS SER ILE ARG ILE GLN ARG GLY PRO GLY ARG
ALA PHE VAL THR ILE GLY

10

3.2. Synthesis

The peptide is synthesized according to the Merrifield solid phase method, giving, after purification by reverse phase 15 hplc, a peptide of 97% purity. The peptide is homogeneous by SDS-PAGE and gel filtration on TSK 2000 column. Its molecular weight is 2800.

20 3.3. Coupling strategy

EMCS, (succinimidyl 6-maleimidyl hexanoate an heterofunctional crosslinker containing a primary amino reactive group N-hydroxysuccinimide (NHS) and a thiol group 25 (maleimide), was chosen.

3.3.1. Activation

The V₃ peptide contains one Lysine group which was activated 30 with EMCS for 30 min at 37°C, pH 7, in a 1:1 to 4:1 ratio.

3.3.2. Coupling to HBs

After elimination of the excess of crosslinker on a G 10 35 column, HBsAg particles were added in a 1:1 ratio with the

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V_3 peptide. The reaction took place overnight at 37°C, pH 6.5 - 7.5.

The conjugate obtained is purified from the unconjugated V_3 peptide by gel filtration on HR 200 column.

3.3.3. Coupling efficiency

As assayed by radioimmunoassay and ELISA, the coupling efficiency was of 0.4 peptide coupled per HBs monomer, which is equivalent to 40 V_3 peptides per HBs particle or to 1 mg V_3 per 25 mg HBsAg.

15 Example C

Antigen-Adjuvant preparations

1. V_3 HBsAg particles in oil water emulsion

20

The vehicle is prepared as follows. To phosphate-buffered saline (PBS) containing 0.4% (v/v) Tween 80 is added 5% (v/v) Pluronic L121 and 10% squalane. This mixture is then microfluidized. For microfluidization, the emulsion is 25 cycled ten times through a microfluidizer (Model M110 Microfluidics Corp., Newton, Mass.). After five passes through the Microfluidizer, the resulting emulsion comprises only submicron particles. 50 µg 3D-MPL is then added to this emulsion. One volume of this emulsion containing 3D-MPL 30 is mixed with an equal volume of twice concentrated V_3 HBsAg and vortex briefly to ensure complete mixing of the components. The final preparation consists of 0.2% Tween 80, 2.5% Pluronic L121, 5% squalane, 50 µg 3D-MPL and 1 µg equivalent V_3 peptide (corresponding to 25 µg HBsAg) in a 35 250 µl injection dose.

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1.2 V₃ HBsAg/Aluminium Hydroxyde plus 3D-MPL

1 µg equivalent V₃ is adsorbed overnight at 4°C on alum corresponding to 0.5 mg equivalents Al³⁺ in 0.25 ml of 150 mM NaCl, 10 mM phosphate buffer pH 6.8. After overnight incubation, the adjuvant preparation is centrifuged and its supernatant removed. An equal volume of adsorption buffer containing 50 µg 3D-MPL is then added to the alum-bound HBsAg - V₃ peptide.

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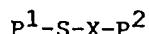
-31-

VACCINES

Claims

5 1. An immunogenic hybrid polypeptide comprising a first polypeptide component (P^1) which is HBsAg or a fragment thereof displaying the antigenicity of HBV surface antigen, covalently linked via a native sulphur atom in the first polypeptide component to a second polypeptide component 10 (P^2).

2. A hybrid polypeptide of the formula



15

in which P^1-S- is HBsAg or fragment thereof displaying the antigenicity of HBV surface antigen bonded via a native sulphur atom;

20

P^2 is a second antigenic polypeptide; and

X is either:

25 a) a group -A-NH- wherein A is a spacer group and NH- is the residue of an amino group present in the side chain of an amino acid in P^2 ;

or optionally if P^2 comprises a cysteine residue and a 30 hydrophobic anchor group:

b) the sulphur atom of the cysteine residue in P^2 .

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3. A hybrid polypeptide as claimed in claim 1 or 2, wherein P² is selected from, gD of Herpes Simplex Virus or a derivative thereof, gp120 from HIV or a derivative thereof, the circumsporozoite antigen or derivative thereof.

5

4. A hybrid polypeptide as claimed in claim 3 wherein P² is gD₂t.

5. A hybrid polypeptide as claimed in claim 3 wherein P² is a peptide corresponding to the V₃ loop of HIV gp120.

6. A hybrid polypeptide as claimed in any of claims 1 to 5 wherein P¹ comprises all or part or parts of the HBs Ag precursor protein.

15

7. A hybrid polypeptide as claimed in any of claims 1 to 6 wherein P¹ is monomer of the particle.

8. A hybrid protein as claimed herein, wherein before fusion P¹ is HBsAg-S-protein having one or more sulphhydryl groups on its surface.

9. A hybrid protein as claimed herein wherein P¹ is a part of a composite particle comprising at least two polypeptides corresponding to part or all of a protein having the biological activity of the hepatitis B surface antigen wherein the particle presents at least two antigenic determinants provided by the S-protein, pre S2-protein or pre S1-protein, said particle optionally containing host specific lipids.

10. A hybrid protein as claimed in claim 9 wherein the composite particle maybe represented by L, S, wherein L is the large protein of HBsAg, including pre S1, pre S2 and S, and S is HBsAg S-protein.

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11. A hybrid protein as claimed in claim 10, characterised is that the L protein comprises one or more of the following modifications in the following sequences:

5 a sequence sensitive to protease digestion,
 a sequence necessary for myristylation,
 a sequence necessary for N-linked glycosylation,
 a sequence necessary for O-linked glycosylation, and
 a sequence necessary for binding of human serum albumin.

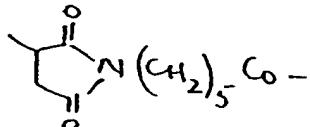
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12. A hybrid protein as claimed in claim 10 or 11, wherein L is modified and has an amino acid sequence comprising residues 12-52, followed by 133-145, followed by residues 175-400 of the L-protein.

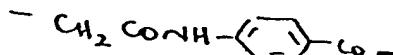
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13. A hybrid protein as claimed in any of claims 2 to 12 wherein A is a substituted C_2-C_{10} alkane or a linear polymer such as polyethylene glycol or a compound

20



25



and



14. A vaccine formulation comprising a hybrid protein as claimed in any of claims 1 to 13 in conjunction with a pharmaceutically acceptable excipient.

15. A vaccine formulation as claimed in claim 14 further comprising de - 3 - O - acylated monophosphorylated lipid A (3D-MPL) in a suitable carrier.

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16. A vaccine formulation as claimed herein comprising 3D-MPL and alum.

17. A vaccine formulation as claimed herein wherein the carrier is an oil in water emulsion.

18. A vaccine formulation as claimed in claim 18 wherein the oil in water emulsion comprising 3D-MPL, squalane, pluronic L - 121 and phosphate buffered saline.

10

19. A process for the preparation of a hybrid polypeptide of formula (I):



15

wherein P^1 , P^2 and X are as defined in claim 2 which process comprises the steps of

a) when X is an a group -ANH-:

20

i) reacting the second polypeptide P^2 with a compound of formula (II):



25

wherein Y is a group capable of reacting with a native thiol group in the first polypeptide; B is a group which is specific for one or more amino acid side chains on the second polypeptide P^2 and A is as hereinabove defined; and 30 thereafter

ii) reacting the product with the first polypeptide P^1-SH ; or

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b) when X is S and P² comprises a hydrophobic anchor group and a cysteine residue:

- i) mixing the first polypeptide and second polypeptide 5 in aqueous solution; and
- ii) allowing an intermolecular disulphide bond between P¹ and P² to form by spontaneous oxidation.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 91/02422

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 C07K15/04; A61K39/29

II. FIELDS SEARCHED

Minimum Documentation Searched?

Classification System	Classification Symbols
Int.C1. 5	C07K ; A61K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁷III. DOCUMENTS CONSIDERED TO BE RELEVANT⁸

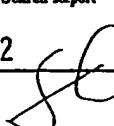
Category ⁹	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹¹	Relevant to Claim No. ¹²
A	EP,A,0 271 302 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 15 June 1988 see page 3, line 10 - line 30 see page 6, line 55 - page 7, line 33 see page 7, line 48 - line 64 see page 11, line 9 - line 21 see page 12, line 54 - page 13, line 25 ----	1-19
A	EP,A,0 326 109 (NEW YORK BLOOD CENTER) 2 August 1989 see page 2, line 28 - page 3, line 37 see page 5, line 35 - page 8, line 15 ----	1-19
A	EP,A,0 385 610 (THE WELLCOME FOUNDATION) 5 September 1990 see page 2, line 38 - page 3, line 20 ----	1-19 -/-

¹⁰ Special categories of cited documents :

- ¹¹ "A" document defining the general state of the art which is not considered to be of particular relevance
- ¹² "E" earlier document but published on or after the international filing date
- ¹³ "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- ¹⁴ "O" document referring to an oral disclosure, use, exhibition or other means
- ¹⁵ "P" document published prior to the international filing date but later than the priority date claimed

¹⁶ "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention¹⁷ "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step¹⁸ "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.¹⁹ "A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 16 MARCH 1992	Date of Mailing of this International Search Report 19. 03. 92
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer KORSNER S.E. 

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>PROCEEDINGS OF THE AMERICAN NATIONAL ACADEMY OF SCIENCE vol. 85, 1988, WASHINGTON pages 1932 - 1936; PALKER ET AL: 'Type-specific neutralization of the human immunodeficiency virus with antibodies to ENV-coded synthetic peptides' * P. 1932 (Abstract) * * P. 1933 (Col. 1, top) * ---</p>	1-19
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE U.S.A. vol. 85, 1988, WASHINGTON pages 4478 - 4482; GOUDSMIT ET AL: 'Human immunodeficiency virus type I neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees' * P. 4478 (Abstract) * ---</p>	1-19
A	<p>JOURNAL OF PROTEIN CHEMISTRY vol. 2, no. 3, 1983, NEW YORK pages 263 - 277; PARTIS ET AL: 'Cross-linking of protein by N-maleimido alkanoyl N-hydroxysuccinimid esters' * P. 267-270 * ---</p>	1-19
A	<p>BIOTECHNOLOGY vol. 3, no. 4, 1985, LONDON pages 323 - 326; VALENZUELA ET AL: 'Antigen engineering in yeast: Synthesis and assembly of hybrid hepatitis B surface antigen-herpes simplex 1 gD particles' * P. 323 * ---</p>	1-19

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. EP 9102422
SA 54130

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 16/03/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0271302	15-06-88	US-A- US-A- AU-A- JP-A-	4882145 4818527 8223187 1025800	21-11-89 04-04-89 09-06-88 27-01-89
EP-A-0326109	02-08-89	US-A-	5039522	13-08-91
EP-A-0385610	05-09-90	AU-A- JP-A-	4975590 3027400	18-10-90 05-02-91

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82